Protracted induction of parturition enhances placental maturation, but does not influence incidence of placntal retention in cows

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Abstract: As the etiopathology of retained placenta is still not resolved in cattle, we compared the effects of protracted induction of parturition (PIP) and conventional induction of parturition (SIP) on placental maturation and the occurrence of retained placenta. PIP was induced in 13 cows by administration of 1.3 mg dexamethasone im twice daily between Days 268 and 273 of gestation and 40 mg dexamethasone im on Day 274 of gestation. For SIP, 10 cows received a single injection of 40 mg dexamethasone on Day 274 of gestation. A third group (SPON, n = 11) served as a nontreated control group. Within 2 hours after birth, two placentomes were extracted from the uterus and used for assessment of placental maturation by histology and immunohistochemistry. Incidence of retained placenta was lower ($P < 0.05$) in group SPON (9%) compared with groups PIP (54%) and SIP (70%). Staining with Masson's trichrome and pan-cytokeratin indicated a higher degree of atrophy and flatness of the maternal crypt epithelium in cows with physiological release of fetal membranes (REL) compared with cows with retained placentae (RET). Staining with anti-caspase-3 ratified the observations as more apoptotic cells were detected in groups SPON and PIP compared with group SIP; however, data were not statistically significant. Additionally, the expressions of the potent vasodilators endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) were evaluated. Both eNOS and iNOS were only expressed in chorionic tissue. Endothelin-1 (ET-1), a major vasoconstrictor, showed positive staining in maternal crypt epithelium and in chorionic epithelium. No differences were found for iNOS and eNOS and ET-1, neither among the experimental groups nor between RET and REL cows. These findings indicate that a PIP results in increased placental maturation, but does not influence the incidence of placental retention in cows. The expression of vasoactive substances does not seem to be related to the placental separation process.

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Short Title: Effects of a protracted induction of parturition in cattle

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Abstract

As the etiopathology of retained placenta is still not resolved in cattle, we compared the effects of protracted and conventional induction of parturition on placental maturation and the occurrence of retained placenta. Protracted induction of parturition (PIP) was induced in 13 cows by administration of 1.3 mg dexamethasone i.m. twice daily between Days 268 and 273 of gestation, and 40 mg dexamethasone i.m. on Day 274 of gestation. For conventional induction of parturition (SIP), 10 cows received a single injection of 40 mg dexamethasone on Day 274 of gestation. A third group (SPON, n = 11) served as a non treated control group. Within two hours after birth, two placentomes were extracted from the uterus and used for assessment of placental maturation by histology and immunohistochemistry. Incidence of retained placenta was lower ($P < 0.05$) in group SPON (9 %) compared to group PIP (54 %) and SIP (70 %). Staining with Masson-trichrome and pan-cytokeratin indicated a higher degree of atrophy and flatness of the maternal crypt epithelium in cows with physiological release of fetal membranes (REL) compared to cows with retained placentae (RET). Staining with anti-caspase-3 ratified the observations as more apoptotic cells were detected in group SPON and PIP compared with group SIP, however data were not statistically significant. Additionally, the expressions of the potent vasodilators endothelial (eNOS) and inducible (iNOS) nitric oxide synthase were evaluated. Both, eNOS and iNOS, were only expressed in chorionic tissue. Endothelin-1 (ET-1) a major vasoconstrictor showed a positive staining in maternal crypt epithelium as well as in chorionic epithelium. No differences were found for iNOS and eNOS as well as ET-1 neither among the experimental groups nor between RET and REL cows. These findings indicate that a protracted induction of parturition results in increased placental maturation, but does not influence the incidence of placental retention in cows.

The expression of vasoactive substances does not seem to be related to the placental separation process.

Keywords: placental retention; placental maturation, induction of parturition, dexamethasone; cattle

1. Introduction

Retention of fetal membranes is still a common problem in dairy cattle which negatively affects for fertility [1-6]. Approximately five to ten per cent of dairy cows suffer from placental retention [2,4]. If induction of parturition is indicated for medical reasons, the incidence of retained placenta increases even further to 80 - 95 per cent [4,5]. Medical reasons for termination of pregnancy are for example a prolonged pregnancy, Hydrallantois/-amnion, mummification, expected high birth weights in calves produced by in vitro maturation and in vitro fertilization [7] or too early, unwanted occupation of young cattle. Additionally it has been shown that stagnation in parturition was the main reason for stillbirth in cows besides specific dystocia, such as malposition and uterine torsion. One method to reduce this problem is to terminate labor via hormonal induction of parturition for a better supervision in calving animals, especially when difficulties are expected.

Although there are many studies concerning retained placentae in cows, the etiology and pathogenesis of this problem are not fully understood. Many factors have been implicated in the development of placental retention, all of them leading to/involving an incomplete placental maturation [8,9]. The maturation of the bovine placenta is usually completed three to five days before parturition [8]. Histologically, it is characterised by flattening of the maternal crypt epithelium and a decrease in height and number of these cells [8,10].

74 Furthermore, an increase in the number of apoptotic cells in maternal and fetal epithelial
75 tissues is found during the maturation process [3,8,9,11].
76 Additionally, placental separation might be facilitated during the series of dilation and
77 contractions of the uterus close to term. The constantly changing uterine pressure leads to
78 alternating ischemic and hyperaemic conditions within the placentomes which could result
79 in an impaired fetomaternal adherence [12]. Nitric oxide (NO), which is synthesized from
80 L-arginine by NO synthases (NOS), is a potent vasodilator in placental tissue [13].
81 Previous studies have shown that NO plays an active role in regulating placental function
82 including reducing the vascular tone during human [14] and ovine [15] pregnancy. Close to
83 term NO-production was found to decrease in humans effectively promoting contractions
84 resulting in labor [16]. There are three isoforms of NOS: neuronal NOS (nNOS), inducible
85 NOS (iNOS) and endothelial NOS (eNOS) [17]. It has been shown that iNOS and eNOS
86 are expressed in the placenta of many species [18,19]. Endothelins are the counterparts to
87 the vasodilatation system and belong to a family of vasoconstrictor peptides, mainly
88 produced by endothelial cells of mammalian species [20]. Especially Endothelin-1 (ET-1)
89 has been shown to be involved in the regulation of uterine and placental perfusion [21] and
90 in the stimulation of the uterine contraction during labor and delivery in rats [22]. The role
91 of these vasoactive substances in reference to placental separation has not been studied
92 until now.
93 Glucocorticoids have been identified to play a central role in the placental maturation
94 process [23,24]. As fetal cortisol levels gradually increase in the course of pregnancy,
95 placental maturation seems to require exposure to elevated cortisol levels prior to calving
96 [23]. Conventional methods of induction of parturition, involving a single application of
97 corticosteroids in the attempt to mimic the natural endocrine events, result in an incomplete
98 placental maturation and therefore in a high incidence of retained placentae [25-28].
99 Several attempts to modify the induction schedule had no significant effect on placental

maturation [26,29-31]. The first promising results in relation to reduction of the incidence of retained placentae came from New Zealand [32]. These authors demonstrated that a pretreatment with long-acting corticosteroids had a positive effect on placental release. However, the laws in the European Union prohibit the exogenous application of long-acting corticosteroids in food animals. As an alternative, the injection of low dosages of short-acting corticosteroids for several days followed by a single application of a high dosage of this hormone has been proposed [33].

In the present study a conventional (single high dose of glucocorticoids) and a protracted (repeated applications of low doses of glucocorticoids followed by a single high dose of glucocorticoids) induction of parturition were compared to determine whether or not repeated applications of low dosages of short-acting corticosteroids for several days result in an improved placental maturation and therefore in a reduced incidence of retained placentae in cows, in which induction of parturition is indicated. Placental maturation was determined histologically and by immunohistochemistry utilizing markers for epithelial cells and apoptosis. The potential involvement of vasoactive substances on placental maturation was assessed by immunohistochemistry for vasodilators and vasoconstrictors.

2. Materials and Methods

2.1 Experimental Design

Twenty-four Holstein Friesian, eight German Black Pied, one German Fleckvieh and one Red Holstein cow were used in this study between June 2007 and December 2008. Cows were 3.2 ± 1.2 years old (range, 2 to 8), with a parity of 1.7 ± 1.0 (range, 1 to 5). Twelve days before their expected calving date, cows were brought into stables with deep-straw bedding and fed a mixed ration (corn, grass silage, ground corn, vitamins and minerals),

126 with *ad libitum* access to water. All cows were controlled every 4 h for signs of an
127 imminent parturition. If cows did not deliver the fetus within 2 h after the first visible signs
128 of labor, the position and size of the fetus was examined by transvaginal manual
129 exploration of the birth canal. If indicated due to the obstetric findings, assistance of
130 parturition was provided.
131 These 34 animals with known breeding dates (Day 0 = day of insemination) were
132 randomly selected for a protracted induction of parturition (PIP, n = 13) and a conventional
133 induction of parturition (SIP, n = 10), while the remaining 11 cows served as the non
134 treated controls (SPON). On Day 268 of gestation, group PIP received 1.3 mg
135 dexamethasone (Dexamethason®, cp-pharma, Burgdorf, Germany) i.m. twice a day for six
136 days. On Day 274, 40 mg dexamethasone i.m. was administered. Group SIP received only
137 a single injection of 40 mg dexamethasone i.m. on Day 274 of gestation. The control group
138 calved spontaneously without any treatments. All injections were administered by the same
139 person (DH) and with minimal stress for the high pregnant animal.
140 Within two hours after birth, two placentomes were extracted from the uterus and used for
141 assessment of placental maturation by histology and immunohistochemistry.
142 The end point variables recorded were the date of parturition, interval from calving to
143 placental release and incidence of placental retention. Cows that expelled the placenta
144 within the first 12 hours were defined as cows with released placentae (REL). Cows that
145 had not expelled their placenta within the first 12 hours after calving were defined as cows
146 with a retained placenta (RET) and were treated every 24 hours with 1.0 mg Ceftiofur per
147 kg body weight s.c. (Excenel® RTU, Pfizer AG, Zürich, Swiss) for five days. In the
148 following days time of separation of the placenta in RET cows was monitored and
149 recorded.
150 Treatment of the cows and removal of placentomes after birth was approved by the local
151 independent authority ensuring animal welfare (LAVES Az: 33.9-42502-04-07/1394).

152

153 *2.2 Tissue sampling and fixation*

154

155 Within two hours after birth two placentomes were collected per vaginam by an elongated
156 effeminator designed by Reisinger, modified by Richter (Company Hauptner, Solingen,
157 Germany). The collected placentomes were cut into radial slices, which were further cut
158 into small pieces of about 1.0 x 1.0 x 0.5 cm. The specimens were immersed in 4 % neutral
159 phosphate-buffered formaldehyde solution according to Lillie [34] for 24 hours and after-
160 wards embedded in Paraplast Plus ® (Shandon, Pittsburgh, PA, USA) using a tissue
161 embedding machine (Tissue-Tek III, from Shandon, Pittsburgh, PA, USA).

162

163 *2.3. Histology and Immunohistochemistry*

164

165 For histology, two sections with a thickness of about 5 µm were produced from a total of
166 26 cows (CON, n = 8; SIP, n = 8; PIP, n = 10) and stained with Masson´s trichrome.
167 For immunohistochemical examinations paraffin sections were dried at 37 ºC overnight,
168 deparaffinized in xylene and rehydrated in a series of graded alcohols at room temperature.
169 All incubation steps were performed in a moist chamber and all dilutions were carried out
170 with PBS (0.1 M, pH 7.2). After quenching the activity of endogenous peroxidase with 4
171 % H_2O_2 in 80 % alcohol for 30 min the sections were rinsed and incubated with normal
172 goat serum for 20 min at room temperature in order to block non specific binding sites.
173 After removal of the blocking serum the slides were incubated with the primary antibody at
174 4 ºC overnight.
175 For visualisation of the immunostaining, the sections were incubated with EnVision rabbit
176 (Dako REAL EnVision Detection System, Glostrup, Denmark) at room temperature for 45
177 min, rinsed with PBS (3 x 5 min) and stained with diaminobenzidine (DAB) chromogenic

substrate (Dako REAL EnVision Detection System, Glostrup, Denmark). Finally, the sections were rinsed in PBS and tap water and counterstained with hematoxylin for 30 seconds.

For Cytokeratin detection a Pan-Cytokeratin antibody, consisting of clones AE1 and AE3 (Bioprime, DNA Labeling System, Gibco BRL Grand Island, NY, USA, CK102) were used as primary antibody in a dilution of 1:100 to identify maternal crypt epithelium and chorionic epithelium respectively. AE1 and AE3 are murine IgG1 antibodies elicited against human epithelial keratins.

Apoptotic cells were identified by a rabbit polyclonal antibody in a dilution of 1:50 against Caspase 3, elicited against recombinant full length of human protein (Abcam, Cambridge, MA, USA, ab4051).

To localise inducible nitric oxide synthase (iNOS) a polyclonal rabbit antibody against the N-terminus of murine iNOS in a dilution of 1:500 (Millipore, Billerica, MA, USA, 06-573) was used. Endothelial nitric oxide synthase (eNOS) was localised by a rabbit anti-bovine eNOS IgG antibody in a dilution of 1:2000 (Alpha diagnostic, San Antonio, TX, USA, ENOS 32-A).

The vasoconstrictor Endothelin-1 (ET-1) was detected in placental tissue, using a primary polyclonal rabbit antibody in a dilution of 1:300 (Biologo, Kronshagen, Germany, EDN001).

Negative controls were set up with the antibody diluent instead of the primary antibodies.

2.4. Light Microscopy

Massons´s trichrome stained sections and all immunohistochemistry stainings were evaluated using an Olympus light microscope BX51 equipped with Olympus DP72 Digital Camera (Olympus Europa GmbH, Hamburg, Germany). Based on this examination they

were assigned into three classes according to Schoon [35]: mature placenta, immature placenta and a hybrid form with mature and immature parts in one section. The classification was done by one person (D. H.) who was blinded to the experimental groups. For Caspase-3 in each section (n = 52) the immunopositive brown staining of 12 defined fields of view was quantified for particle size (200x magnification), using analySIS® Soft Imaging System 3.2 (Build 635, Olympus Europa GmbH, Hamburg, Germany).

2.5. Statistical Analysis

Statistical analysis for immunopositive brown staining of Caspase-3 was carried out using SAS® computer program (SAS Inst. Inc., Version 9.1, Cary, NC). Means ± SD were calculated for all measurements (PROC MEANS). For comparison between the experimental groups PIP, SIP and SPON and between RET and REL data were subjected to Student´s *t* - test. Classification of placental maturity (mature, immature and hybrid) was compared between groups PIP, SIP and SPON and between RET and REL using Chi-square distribution. Differences were considered statistically significant when P values were d 0.05.

3. Results

3.1. Clinical findings

Cows of group SPON showed a gestation length of 282 ± 4.1 days (range 278 to 289 days, n = 11). Animals of group SIP had a gestation length of 276 ± 0.4 days (range 276 to 277 days; n = 10) and calved 30 to 70 (46.2 ± 6.9) hours after the injection of 40 mg dexamethasone. Cows of group PIP showed a gestation length of 275 ± 0.95 days (range

272 to 276 days, n = 13). Out of these, nine cows calved between 24 and 36 (27.9 ± 4.8) hours after the last injection of 40 mg dexamethasone. Three cows calved before the last injection of 40 mg dexamethasone was given between Day 272 and 274 and one cow calved on Day 276.

Obstetrical assistance was required in two cows of groups SPON, SIP, and PIP, respectively. Four cows (SPON: 1, SIP: 2, PIP: 1) needed traction force of one person and one cow (SPON) needed traction force of two people. In one cow (PIP), the position of the calf with the head tucked back had to be corrected.

One out of 11 cows in group SPON (9 %), seven out of 10 (70 %) in group SIP and seven out of 13 cows (54 %) in group PIP had retained placentae. Incidence of retained placenta was significantly lower in group SPON compared with group PIP and SIP ($P < 0.05$). No differences ($P > 0.05$) were found between group PIP and SIP. Separation of the placenta in RET cows occurred 3 to 8 days after parturition. All cows with RET showed a metritis of grade I as defined by Sheldon et al. [36]. Effects of breed could not be determined, because the number of animals of some breeds was too small.

In group SPON two calves died, one during the calving process because of prolonged and difficult labor (traction force of two people for more than 30 minutes) and one was already dead, when the cow was examined to check the presentation of the calf in the birth canal. In groups SIP and PIP no calf died, but two and one, respectively, needed nursing assistance.

3.2. Placental Histology (Masson´s Trichrome staining)

Eighteen of 49 analyzable sections were assigned to/identified as a mature placenta form, with obvious signs of loosening of the fetomaternal adherence. In addition, most of the maternal crypt epithelium appeared flattened or was partly inconsistent (Fig.1 A). In 18 of

49 sections, the placentae were clearly immature as the cotyledonary villous trophoblast cells were in intimate contact with an intact maternal caruncular epithelium (Fig.1 B). Thirteen of 49 sections had a hybrid placenta form, where both forms appeared together.

Decoding of the assigned histologic sections revealed that most of the sections (68 %) from animals of group SPON were classified into the mature form, whereas most of the SIP cows had an immature placental structure (71 %). Cows of group PIP predominantly had a hybrid form (42 %) (Fig. 3).

Sixty-seven percent of cows with RET had an immature placenta, eight percent a mature placenta and 25 % showed a hybrid form. In REL cows, 66 % had a mature placenta, 21 % an immature and 14 % a hybrid placenta form ($P < 0.05$).

3.3. Height of Maternal Epithelium (Pan-Cytokeratin Immunohistochemistry)

In tissue sections the intensity of the immunopositive brown staining differed between the chorionic and the maternal crypt epithelium, thus a clear identification of the maternal epithelium was possible (Fig.1 C and D). The assignment to mature and immature placentae completely coincided with the classification of the Masson trichrome sections.

Sections, which were classified as mature in Masson trichrome staining, showed an almost flat and broad maternal crypt epithelium with partly atrophic areas (Fig. 1 C). Cows with an immature placenta showed an almost cuboidal maternal epithelium (Fig. 1 D).

3.4. Apoptosis (Caspase-3 Immunohistochemistry)

In tissue sections Caspase-3 positive cells were found in the fetal and the maternal compartment and showed a brown staining which was mostly localised to the cytoplasm (Fig.1 E and F). In sections, which were classified as mature in Masson's trichrome,

microscopically a greater number of apoptotic cells was detected (Fig.1 E), whereas cows with an immature placenta showed only a few apoptotic areas (Fig.1 F). This revealed that cows of the control group had a slight drift to higher numbers of apoptotic cells (2.31 ± 0.45 %) compared to the conventional induction group (1.68 ± 1.13 %), whereas cows with a protracted induction of birth showed numbers between the controls and the conventional induction group (1.93 ± 0.67 %).

However, the differences were not statistically significant, neither between experimental groups nor between REL and RET cows.

3.5. iNOS and eNOS in placental tissue

Positive staining for iNOS and eNOS was detected in all three groups, but exclusively in fetal placental tissue (Fig. 2 A and B). No positive staining was present in maternal crypt epithelium and maternal stroma cells. Endothelial cells of blood vessels were also positive for eNOS, whereas they were negative for iNOS. No microscopic differences in staining intensity were found between the experimental groups and RET and REL cows.

3.6. Endothelin-1 (ET-1) in placental tissue

In tissue sections a positive staining for ET-1 was found in maternal crypt epithelium as well as in chorionic epithelium (Fig. 2 C). Among the experimental groups and between RET and REL cows, ET-1 immunoreactions were observed in the same cell populations and showed the same staining intensity.

4. Discussion

For the first time we have shown that a protracted induction of parturition leads to an accelerated placental maturation if short-acting corticosteroids are administered twice daily over a period of six days.

Parturition is normally induced by the release of cortisol from the fetus in the last month of gestation, particularly in the last week [37,38]. Cortisol stimulates the enzyme 17±-hydroxylase in the fetal membranes to catalyze the conversion of progesterone to estrogens [39,40]. It is recognized that the magnitude of the prepartum surge in estrogens greatly influences the placental maturation process [41]. Conventional induction of parturition by dexamethasone injection is thought to mimic the physiological mechanisms by which the fetus induces parturition. But a single dose of dexamethasone seems to have no positive effect on the incidence of retained placentae in cows [42,43]. To obtain an improved placental separation fractionate doses of corticosteroids over three days followed by an injection of prostaglandin have been tested [28]. This treatment resulted in a more predictable calving time and therefore in a better supervision of the calving animals, but had no positive effect on placental separation. However, previous studies determined a minimum pretreatment time of five days with corticosteroids to obtain a positive effect on the separation process [23,27,30,38,44]. In the present study a pretreatment time of six days were chosen. The results showed that 54 % of the group with protracted induction of parturition had a retained placenta compared to the group with conventional induction of birth with 70 % of retained fetal membranes, however data were not significant.

The cost of repeated treatments with low doses of corticosteroids for six days followed by one single treatment with a high dose of this hormone is approximately €120. In comparison the economic cost of a single case of metritis, which occurs in many cases after dystocia has been calculated to be about €292 [45].

The delivery of the placenta post partum is a physiological process, involving the loss of fetomaternal adherence [46]. This loss of adherence occurs only after the placentome has undergone a process of maturation, which is initiated several weeks before parturition and not completed until the last days before term [8].

In the present study, the degree of bovine placental maturation, assessed by general histology and immunohistochemistry for pan-cytokeratin and caspase-3, was obvious by separation of fetal and maternal tissues, flattening and disappearance of maternal crypt epithelium, and abundance of apoptosis. Masson´s trichrome staining showed that most of the SPON cows had a mature placenta with a flattened and discontinuous maternal crypt epithelium. This observation concurred to most of REL cows. In contrast most of the animals of group SIP showed an incomplete maturation what in turn concurred to most of RET cows. Cows of group PIP moved in between and showed predominantly a hybrid form between a mature and an immature placenta. These observations were confirmed by an immunohistochemical staining with Pan-Cytokeratin, which not only stained epithelial cells, but allowed also a clear differentiation between maternal crypt epithelium and chorionic epithelium. Maternal and fetal epithelial cells differed clearly in their staining intensity permitting an assessment of the cell height. It has been suggested before that the maturation process involves the flattening of the maternal crypt epithelium and a decrease in the number of maternal epithelial cells [8,47].

In the present study the extent of apoptosis between the experimental groups was evaluated semiquantitatively in caspase-3 immunohistochemically stained sections. No differences between REL and RET cows were found. Animals of Group SPON had a marginal increased occurrence of apoptotic cells compared to Group PIP. Fewest apoptotic cell areas were found in cows of Group SIP. However data are not significant.

Normal pre-term maturation of the bovine placenta has also been associated with apoptosis within the maternal crypt as well as in the chorionic epithelium [3,9,10,48]. However earlier studies are partly contradictory in the occurrence of apoptotic and necrotic cells in placental tissues. Some studies found that placental necrosis was not directly related to normal placental separation of the fetal membranes from the maternal caruncle [3,48]. Others detected an increase in the number of apoptotic cells in maternal and fetal epithelium immediately after the expulsion of the fetus and correlated these observation with the placental maturation process [10]. In contrast, Boos et al. [9] found more apoptotic cells in animals retaining their fetal membranes than in cows with placental release.

Another cause of fetal membrane retention is assumed to be the maintenance of blood pressure within the chorionic villi. Contractions of the myometrial smooth muscle layers lead to alternating hyperaemic and ischaemic conditions within the fetal villi [12]. Thus the constantly changing uterine pressure after maturation impairs the feto-maternal junction and seems to be of great importance for separation of the bovine placenta [6]. To elucidate the additional potential impact of vasoactive substances we examined the presence of a vasoconstrictor as well as vasodilatators by means of immunohistochemistry.

In the present study antibodies against iNOS and eNOS were used to detect NO production in the bovine placenta in the last days of pregnancy. We found that iNOS and eNOS are expressed in all bovine placental tissue sections. But differences were found neither in the exhibition of iNOS and iNOS among the experimental groups nor between REL and RET cows. As expected eNOS staining was observed in the endothelium of all blood vessels, whereas iNOS was not expressed. Surprisingly, unlike in sheep where iNOS was localized in intercotyledonary chorioallantoic membrane and intercaruncular maternal endometrium and eNOS was present in cotyledons and caruncles of the placentome as well as in

384 intercotyledonary and intercaruncular placental tissue [49], positive staining for iNOS and
385 eNOS was only detected in fetal placental tissue. This finding implicates that the fetal
386 chorionic tissue of the bovine placenta has a more active part in placental vasodilatation
387 than previously thought.
388 In the present study the localization of ET-1 peptide in bovine placentomes was described
389 for the first time. ET-1 was found in maternal crypt epithelium as well as in chorionic
390 epithelium. Like for iNOS and eNOS no differences in the expression of ET-1 were found
391 between the experimental groups or between REL and RET cows. Therefore ET-1 and
392 NOS could not be correlated with placental maturation and placental retention, which is in
393 accordance with previous studies [50,51]. In bovine caruncles and cotyledons ET-1 mRNA
394 is expressed during the entire pregnancy [50]. However the authors found no correlation
395 between ET-1 protein expression in placental tissues and placental retention.
396 The finding that vasoconstrictive ET-1 and vasodilatory iNOS and eNOS did not differ
397 between the groups examined is supported by results of parallel uterine blood flow studies,
398 which showed no differences in blood flow volume and resistance index in the last days
399 before parturition (Hartmann et al., unpublished data).
400
401 In conclusion a protracted induction of parturition with repeated applications of low
402 dosages of dexamethasone over six days as a pretreatment for a conventional induction
403 treatment might mimic the physiological mechanisms by which the fetus induces
404 parturition in cattle. This leads to a better cellular placental maturation, although it does not
405 influence incidence of retained placenta.
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407
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409

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List of figures

Figure 1: Illustration of mature (REL) and immature (RET) bovine placentomes.
A, B Masson trichrome staining in mature (A) and immature (B) placental formation. **A** Asterisks (*) illustrate the loosening of the feto-maternal adherence. **B** In immature placentae the fetal and maternal part are tightly adhered to each other. **C, D** Immunohistochemical staining with Pan-Cytokeratin. **C** The maternal crypt epithelium appears flattened or is partly inconsistent (arrows). **D** Illustration of an almost cuboidal maternal crypt epithelium in RET cows (arrowheads). **E, F** Immunohistochemical staining with Caspase-3 evidenced a greater number of immunopositive brown staining cells in mature (**E**) and only scattered positive cells in immature (**F**) placental tissue sections. **fe**: fetal chorionic epithelium, **me**: maternal crypt epithelium. Bars 100 µm.

Figure 2: Formation of nitric oxide synthase (NOS) and Endothelin-1 (ET-1) in bovine placentomes. **A, B** Immunohistochemistry staining with eNOS (**A**) and iNOS (**B**) showed a clear appearance of positive staining only in fetal chorionic tissue. **C** Positive staining for ET-1 is present in fetal chorionic tissue as well as in maternal crypt epithelium in bovine placental tissue. **D** Negative immunohistochemistry control. **fe**: fetal chorionic epithelium, **me**: maternal crypt epithelium. **A**-**C** Bars 50µm, **D** Bars 100µm.

Figure 3: Classification of placental formation in the experimental groups at delivery: control (SPON, n = 8), conventional induction of parturition (SIP, n = 8) and protracted induction of parturition (PIP, n = 10).

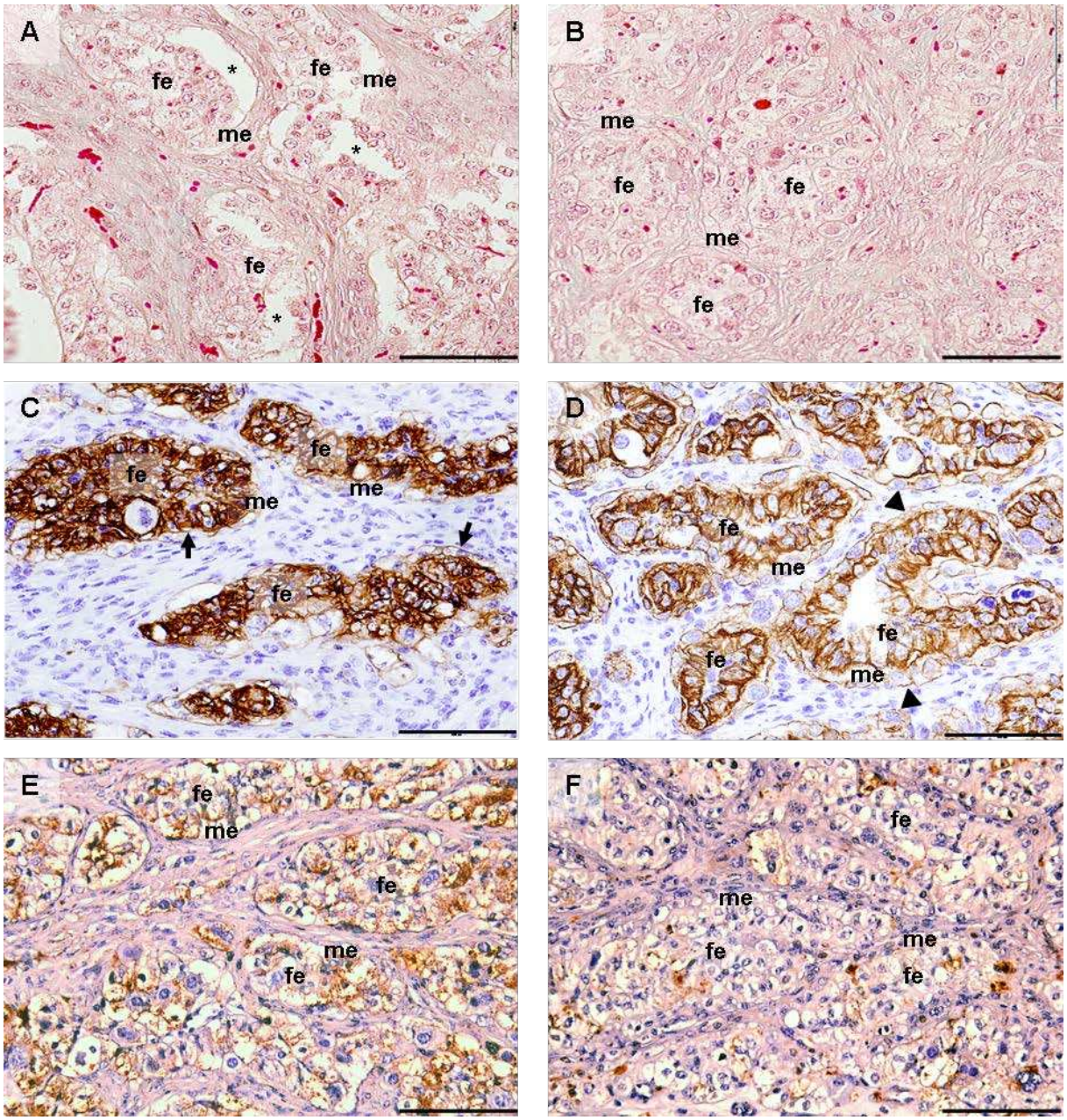
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Figure 1

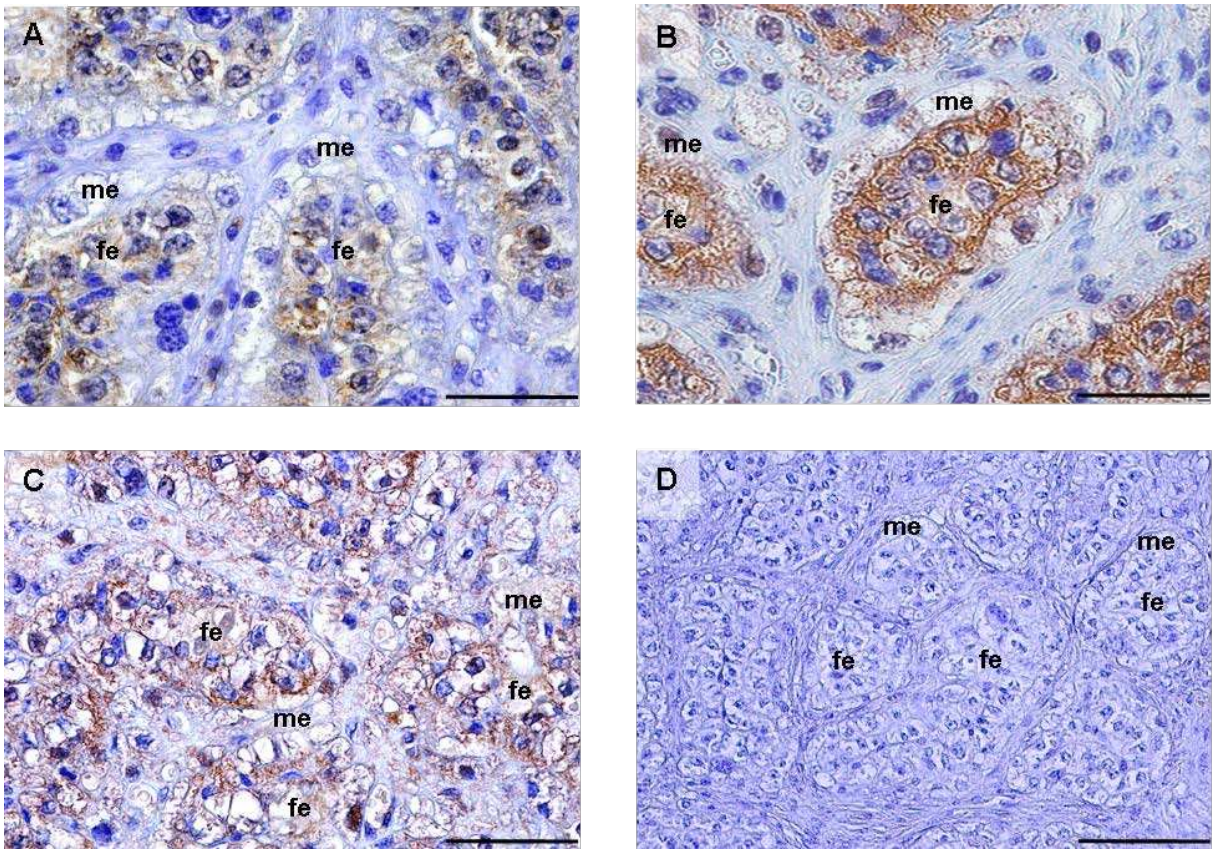

Figure 2

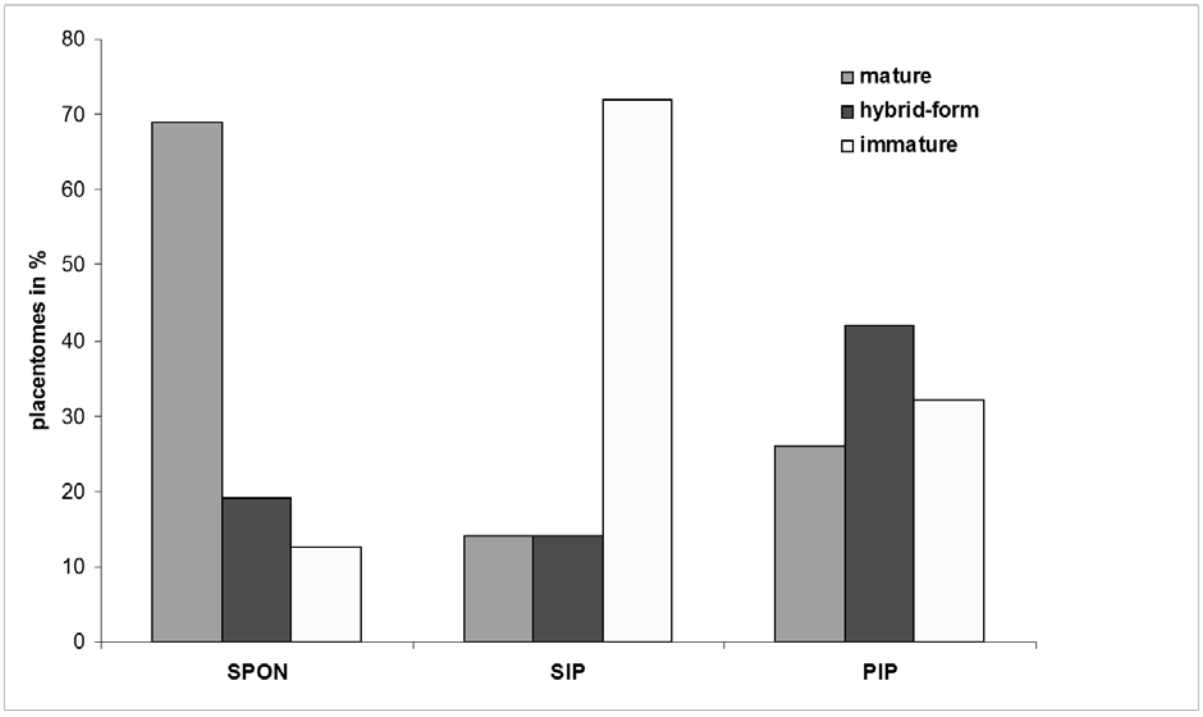

Figure 3